

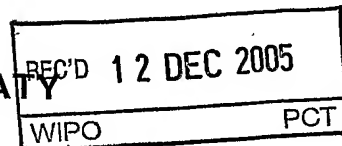
PATENT COOPERATION TREATY


PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference HMJ03737WO	FOR FURTHER ACTION	See Form PCT/PEA/416
International application No. PCT/GB2004/003488	International filing date (day/month/year) 12.08.2004	Priority date (day/month/year) 12.08.2003
International Patent Classification (IPC) or national classification and IPC C08B37/00, C07K1/07, A61K47/48		
Applicant LIPOXEN TECHNOLOGIES LIMITED et al		
<p>1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 7 sheets, including this cover sheet.</p> <p>3. This report is also accompanied by ANNEXES, comprising:</p> <p>a. <input checked="" type="checkbox"/> sent to the applicant and to the International Bureau) a total of 21 sheets, as follows:</p> <p><input checked="" type="checkbox"/> sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).</p> <p><input type="checkbox"/> sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.</p> <p>b. <input type="checkbox"/> (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).</p>		
<p>4. This report contains indications relating to the following items:</p> <p><input checked="" type="checkbox"/> Box No. I Basis of the opinion</p> <p><input type="checkbox"/> Box No. II Priority</p> <p><input type="checkbox"/> Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p><input type="checkbox"/> Box No. IV Lack of unity of invention</p> <p><input checked="" type="checkbox"/> Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p><input type="checkbox"/> Box No. VI Certain documents cited</p> <p><input type="checkbox"/> Box No. VII Certain defects in the international application</p> <p><input checked="" type="checkbox"/> Box No. VIII Certain observations on the international application</p>		
Date of submission of the demand 14.03.2005	Date of completion of this report 09.12.2005	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Radke, M Telephone No. +49 89 2399-8677	



INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2004/003488

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.
- ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
- ☐ international search (under Rules 12.3 and 23.1(b))
 - ☐ publication of the international application (under Rule 12.4)
 - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)
2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-18 received on 14.03.2005 with letter of 11.03.2005

Claims, Numbers

1-30 received on 14.03.2005 with letter of 11.03.2005

Drawings, Figures

1, 2 as originally filed

☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
- ☐ the description, pages
 - ☐ the claims, Nos.
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):
4. ☒ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
- ☒ the description, pages 17
 - ☒ the claims, Nos. 1-30
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):

* If item 4 applies, some or all of these sheets may be marked "superseded."

**INTERNATIONAL PRELIMINARY REPORT
ON PATENTABILITY**

International application No.
PCT/GB2004/003488

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	14,15
	No: Claims	1-13,16-22
Inventive step (IS)	Yes: Claims	
	No: Claims	14,15
Industrial applicability (IA)	Yes: Claims	1-22
	No: Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I

Basis of the report

1. The applicant has replaced the following feature in claims 1 and 11 (to form claims 1 and 14 as filed with the letter dated 11.03.2005):

"a polysaccharide acid" by

"a polysaccharide having at least two sialic acid units joined to one another".

There is no basis for this amendment in the application as originally filed. Original claim 10 only discloses a polysaccharide having at least two sialic acid units **2,8 and/or 2,9** linked to one another.

2. The applicant has inserted the following feature in claim 8 at page 20, line 8:

"R³ is H;"

There is no basis for this amendment in the application as originally filed.

3. Furthermore, the following amendment has no basis in the application as originally filed:

The deletion of "EDTA" at 17/27.

4. These amendments thus introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 19(2)/Article 34(2)(b) PCT.
5. As the only independent claims contain added subject-matter, claims 1 to 30 as filed with the letter dated 11.03.2005 were not taken into account and examination was carried out on claims 1 to 22 as originally filed

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Cited literature

- (a) The following documents (D) are referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D1: US-A-5 329 028

D2: D. Macmillan et al., Chemistry And Biology, Current Biology, London, GB (2001), 8(2), 133-145

D3: Database Derwent WPI; AN: 1980-23224C &
SU-A-675 053

D4: WO-A-01 87 922

- (b) In the following arguments, page or column A, lines B to C will be cited as A/B-C.

2. Novelty

- (a) Document **D1** describes the coupling of 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH) to the sialic acid residues of soluble CD4 glycoprotein (see 2/14-16; 6/28-33). The sialic acid portion of CD4 is oxidised by means of NaIO_4 and the hydrazide group of MPBH reacts with the aldehyde group of the oxidised CD4. The product thus obtained can react with thiol groups of proteins (see 6/28-45 and fig. 1). As an alternative to MPBH, a hydrazide functional pyridyl disulphide is mentioned (see 6/21-26).

Therefore, the subject-matter of **claims 1-13 and 16-22** is not novel.

- (b) Document **D4** teaches to oxidise polysialic acid (colominic acid) with periodate in order to convert the terminal $-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2\text{OH}$ group into a $-\text{C}(\text{H})=\text{O}$ group (see Fig. 1) and to couple this terminal aldehyde group by reductive amination with lysyl units of a protein (see claims 1, 10, 11 and 16; 6/24-7/6, 8/5-15, 10/6-11/2 and example 4).

The product thus formed is one according to present claim 7, alternative ii) where $\text{R}^1=\text{H}$, $\text{R}^2=-\text{NR}^6\text{R}^7$, where $\text{R}^7=\text{H}$.

So, the subject-matter of **claims 7 and 8** is also not novel in view of **D4**.

3. Inventive step

- (a) Document **D2** discloses in Fig. 1 the reaction of a cysteine unit of a protein with an alpha-iodoacetamido sugar. It is mentioned that this reaction is selective (see p. 134, right col., 2nd para.).
- (b) It was quite obvious to prepare this alpha-iodoacetamido sugar by condensation of the amino group of the sugar with iodoacetic acid in the presence of a carbodiimide.

For instance **D3** discloses to condense the amino group of methyl(-9-anthryl)-amine with iodoacetic acid in the presence of dicyclohexyl-carbodiimide.

- (c) Therefore, it was obvious to modify the process described in **D1** by reacting an amino group of the polysaccharide with iodoacetic acid.

So, the subject-matter of **claims 14 and 15** is not based on an inventive step.

Re Item VIII

Certain observations on the international application

1. Clarity of the claims (Art. 6 PCT)

- (a) **Claim 1** is unclear. There seems to be at least one word missing in the expression "... comprising a polysaccharide a pendant moiety linked at least one terminal unit ...".
- (b) **Claim 7** is unclear since the radical R^9 is not defined in definition ii).

2. Objections under Art. 5 (lack of disclosure) and 6 PCT (support of the claims by

the description)

- (a) The present description is silent as to how most of the compounds of **claim 7** may be produced.

So, there is no information in the description how the compounds of the alternative I) may be made, i.e. compounds where a group R^3 (i.e. $-\text{CH}_2\text{CHR}^4\text{R}^5$ or $-\text{CH}(\text{CH}_2\text{OH})\text{CHR}^4\text{R}^5$) is bonded to a glycosyl group via an oxygen atom.

Likewise, the description does not give any indication as to how compounds of the alternative ii) may be obtained, where R^6 and R^7 together are a 1,3-but-2-enediol group.

- (b) Therefore the person skilled in the art is "... unable ... to extend the particular teaching of the description to the whole of the field claimed ..." (see the PCT Guidelines 5.44) so that **claim 7** is not supported by the description, contrary to the requirements of Art. 6 PCT.
- (c) In addition to that the description does not - as far as the teaching of claim 7 is concerned - "disclose the invention in a manner sufficiently clear and complete for the invention to be carried out by a person skilled in the art." (Art. 5 PCT).
3. The preferred features in **claims 6, 10, 12, 14, 15, and 20** denoted by the word "preferably" represent features that do not limit the claims. They are thus obviously irrelevant (Rule 9.1(iv) PCT) and render these claims inconcise, contrary to the requirements of Article 6 PCT.
4. The last claim should bear the number 22 instead of 20.

POLYSIALIC ACID DERIVATIVES

The present invention relates to polysialic acid derivatives which are useful for conjugation to drugs, proteins and peptides, or to drug delivery systems such as liposomes, having sulfhydryl groups, as well as to
5 conjugated products process for synthesising the derivatives and the conjugates and novel synthetic intermediates.

The extended presence of drugs either within the vascular system or in extravascular use is often a pre-requisite for their optimal use. Many antibiotics and cytostatics for instance, as well as a variety of therapeutic
10 peptides and proteins, and liposomes are removed from the circulation prematurely and before effective concentrations in target tissues can be achieved. The half lives of a number of short-lived proteins (for instance enzymes, cytokines, etc) have been augmented by conjugating these to poly(ethylene glycol). It appears that PEG molecules prolong the circulation
15 time of proteins and particles by forming a cloud around their surface, thus sterically hindering interaction with factors responsible for their clearance. However PEG is non-biodegradable and accumulation of PEGylated proteins intracellularly may be undesirable especially on chronic use [Bendele, A., Seely, J., Richey, C., Sennello, G., Shopp, G., Renal
20 tubular vacuolation in animals treated with polyethylene-glycol conjugated proteins, Toxicological sciences, 42 (1998) 152-157; Convers, C. D., Lejeune, L., Shum, K., Gilbert, C., Shorr, R.G.L, Physiological effect of polyethylene glycol conjugation on stroma-free bovine hemoglobin in the conscious dog after partial exchange transfusion, Artificial organ, 21 (1997)
25 369-378].

We have described the conjugation of a polysaccharide comprising 2→8 and or 2→9 (e.g. alternating 2→8 and 2→9) linked sialic acid units conjugated to proteins to increase their half life, reduce their immunogenicity/antigenicity or increase the stability of a variety of proteins.
30 In WO92/22331, polysialic acids are reacted with a model drug and shown to extend the half life in the circulation of mice. In Cell. Mol. Life Sci. 57

(2000) 1964 to 1969 and in *Biotechnol. Genet. Eng. Rev.* 16 (1999) 203 to 215, Gregoriadis *et al.* describe the conjugation of polysialic acids to asparaginase and catalase, and show that the clearance rates from circulation reduced whilst enzyme activity was retained. We have also polysialylated insulin (*Biochim. Biophys. Acta* 1622 (2003) 42-49 and shown it to be active. We have also polysialylated interferon (AAPS Annual meeting 2002, Toronto, Canada, M1056). We have also polysialylated antibody fragment Fab (Epenetos, A. *et al.*, Proceedings of ASCO (Clinical Pharmacy) 21 (2002) 2186).

In all of these publications, polysialic acid is rendered reactive, by generating an aldehyde group at the non-reducing end by oxidation of the 7, 8-vicinal diol moiety with sodium periodate. The aldehyde group was then reacted with primary amine groups on proteins, generally assumed to be epsilon-amino groups of lysine moieties of the protein or N-terminal amine groups. The reaction forms a Schiff base which is reduced by cyanoborohydride to a secondary amine.

In WO-A-01/87922 we also suggest that derivatisation with other molecules could be carried out in the presence of denaturant to achieve increased levels of derivatisation. Examples of other derivatising agents are polyethylene glycol compounds. Activated PEG compounds such as tresyl-PEG and succinimidyl succinate ester of PEG were mentioned. The examples used succinimidyl succinate activated PEG, which is believed to react with amine groups.

PEG derivatives having functional groups for coupling to thiol groups are commercially available. The functional groups may be maleimide, vinyl sulfone, iodoacetamide or orthopyridyl disulphide groups. Since these reagents react specifically with cysteines, and since proteins have fewer cysteines on their surfaces than lysine groups, the derivatisation is more controllable. Furthermore, in the absence of a free cysteine in a native protein, one or more free cysteines may be added by genetic engineering. The advantage of this approach is that it makes possible site-specific

derivatisation at areas on the protein which will minimise a loss in biological activity.

PEGylated proteins have been found to generate anti PEG antibodies that could also influence the residence time of the conjugate in the blood circulation (Cheng, T., Wu, M., Wu, P., Chern, J, Roffer, SR., Accelerated clearance of polyethylene glycol modified proteins by anti-polyethylene glycol IgM. Bioconjugate chemistry, 10 (1999) 520-528). Despite, the established history of PEG as a parenterally administered polymer conjugated to therapeutics, a better understanding of its immunotoxicology, pharmacology and metabolism will be required (Hunter, A. C; Moghimi, S. M., Therapeutic synthetic polymers: a game of Russian Roulette. Drug Discovery Today, 7 (2002) 998-1001; Brocchini, S., Polymers in medicine: a game of chess. Drug Discovery Today, 8, (2003) 111-112).

It would be useful for modification by polysialic acid to be targeted towards thiol (sulfhydryl) groups. It would also be desirable for the efficiency of derivatisation by sialic acid to be increased, the processes described in our prior art mentioned above requiring high excesses of active polysialic acid. It would also be desirable to avoid the use of cyanoborohydride.

According to the present invention there is provided a novel compound comprising a polysaccharide having a moiety linked at one or each terminal unit which includes a functional group selected from N-maleimido groups, vinylsulphone groups, N-iodoacetamide groups and orthopyridyl disulphide groups.

The terminal unit to which the moiety is linked may be at the non-reducing end of the polysialic acid or at the reducing end of the polysialic acid. Generally the terminal sialic acid unit has been subjected to a preliminary chemical reaction to generate useful functional groups to which a maleimide-group containing reagent may be linked. We have found it convenient to use the chemistry disclosed in our earlier publications in which an aldehyde group is generated, as a preliminary step to generate the functional group via which the maleimide moiety may be linked.

In our earlier publications mentioned above, it is the non-reducing terminal unit which is converted into an aldehyde moiety by oxidation of the 7, 8-vicinal diol moiety with sodium periodate to form the carbon 7-aldehyde compound. This is an appropriate reaction for the present invention.

5 As an alternative, it is possible to provide the aldehyde moiety at the reducing terminal unit. In this case, it is preferred (but is not essential) to carry out a preliminary step in which the non-reducing end is deactivated, by preliminary oxidation and reduction steps. A first reduction step converts the ketal unit at the reducing end into a reduced ring opened form, having vicinal
10 diols. The vicinal diols are subsequently oxidised using sodium periodate to form an aldehyde moiety at the carbon atom previously forming the 7-carbon of the reducing terminal unit. Where the non-reducing terminal glycosyl (usually sialic acid) is not deactivated by a preliminary oxidation step, the terminal unit will be simultaneously activated.

15 In the invention, the moiety which includes the functional group, may be linked directly to the polysialic acid unit or, more conveniently, may be linked via a difunctional organic group, such as an alkane diyl group, an arylene group or an oligo(alkyleneoxy) alkane group, or alternatively an oligo peptidyl group. The linkage to the polysialic acid (from the linker or the
20 moiety including the functional group may be a secondary amine, a hydrazone, an alkyl hydrazine an ester or a peptidyl group. The moiety may be generated by reaction of a polysialic acid substrate with a heterobifunctional reagent. The process form a further aspect of the invention.

25 Reagents useful for introducing the selected functional groups are commercially available. A compound which will introduce a maleimide group onto an amine moiety without introducing any additional linker moiety is N-methoxy-carbonyl-maleimide. Generally the reagents include a second functional group for reaction with a group on the polysialic acid which may
30 be the aldehyde group described above, or an amine group. Suitable second functional groups include N-hydroxy succinimide esters and their

sulfosuccinimide analogues and hydrazides. Preferably the compound is a N-maleimido-alkanoic acid hydrazide or an N-maleimidoarylalkanoic acid hydrazide i.e. a compound having the general formula

X-R-Y in which:

5 X is a N-maleimido, N-iodoacetamido, S-vinylsulphonyl or S-orthopyridyldisulphide group,

R is alkane-diyl, arylene or aralkylene alkarylene, alkylene-oxaalkylene, or alkylene-oligooxa-alkylene or alkyl-oligopeptidyl-alkyl group; and

10 Y is a hydrazide, amine or N-hydroxysuccinimide group.

Preferably R is C₁₋₅ alkanediyl, C₂₋₃-alkyl-oxa-C₂₋₃-alkylene, C₂₋₃ alkyl-oligo(oxa-C₂₋₃ alkylene), or C₂₋₆ alkylene phenyl.

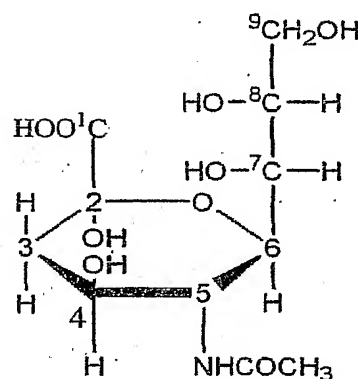
Preferably X is N-maleimido or orthopyridyldisulphide.

Preferably Y is a hydrazide or a hydroxyl succinimide

15 Compounds which may be reacted with an aldehyde group, and which include a linker moiety and introduce a maleimide group include N-[β-maleimidopropionic acid] hydrazide and 4-(4-N-maleimidophenyl)butyric acid hydrazide. The hydrazide group reacts with the aldehyde to form a stable hydrazone group. A suitable heterobifunctional compound which includes
20 an oligo(ethyleneoxy) ethylene group is a compound comprising a polyethylene glycol (poly(ethylenoxy)) group with, at one end, N-hydroxy succinimide (NHS) group and at the other end the functional group. The NHS group reacts with amine groups to form stable amide linkages. Heterobifunctional polyethyleneglycols with NHS at one end and either
25 vinylsulphone or maleimide at the other end are available. Other examples of heterobifunctional reagents include, 3-(2-pyridyldithio)propionyl hydrazide, N-succinimidyl-3-[2-pyridyldithio]propionate, succinimidyl-H-[N-maleimidomethyl]cyclohexane-1-carboxylate, m-maleimidobenzoyl-N-hydroxysuccinimide ester, N-succinimidyl-[4-iodoacetyl] amino benzoate, N-
30 [gamma-maleimidobutyryloxy]succinimide ester, N-[epsilon-maleimidocaproxy]succinimide ester and N-succinimidyl iodoacetate.

Other reagents are available from Pierce Biotechnology and Shearwater Corporation (polyethylene glycol-based).

Sialic acids (also known as nonulosonic acids) are members of a family of amino containing sugars containing 9 or more carbon atoms. The most important of the sialic acids is N-acetylneuraminic acid (also known as 5-(acetylamino)-3,5-dideoxy-D-glycero-D-galacto-nonulosonic, lactaminic acid and O-sialic acid) which has the formula:



Polysialic acids may be linked 2-8 and/or 2-9, usually in the α -configuration. In some polysialic acids the linkages are alternating 2 \rightarrow 8 and 2 \rightarrow 9. The invention is also of utility for heteropolymeric polysaccharides comprising glycosyl units other than sialic acid units.

Polysialic acids are generally found to be non-toxic and substantially non-immunogenic. Furthermore the biodegradation units, sialic acid, is not known to be toxic and, indeed sialic acids are widely found in animal proteins and cells, including blood cells and circulating proteins.

Polysaccharide compounds containing many sialic acid units are polysaccharides produced by *Escherichia coli*, *Moraxella nonliquifaciens*, *Pasteurella aeruginosus* and *Neisseria meningitidis* or derivatives thereof. For instance colominic acid derived (by hydrolysis to shorten the chain lengths) from *E. coli* K1 comprises α 2-8 linked sialic acid units.

Polysaccharide from *E. coli* K92 strain comprises alternating 2-8 and 2-9

linked sialic acid units. Polysaccharide C of *N. meningitidis* group C has 2-9 linked sialic acid units.

One group of polysaccharide compounds which has been found to be of particular utility in the invention is group B polysaccharides. These compounds are produced by *N. meningitidis*, *M. nonliquifaciens*, *P. aeruginosa* A2 and *E. coli* K1. These compounds comprise a polysaccharide component comprising sialic acid residues and a phospholipid component. The sialic acid residues are linked α 2-8 the naturally occurring polymer consisting of about 200 residues. Some of the glycolipid molecules, especially the high molecular weight compounds appear to have a covalently attached phospholipid at the reducing end of the polysaccharide component.

It is preferable for the bacteria from which the polysaccharide compound is derived to be non-pathogenic for convenience during production. It is particularly suitable therefore for the polysaccharide to be derived from a non-pathogenic strain of *E. coli* such as *E. coli* K92 or, preferably, K1 which is non-immunogenic. *E. coli* K92 and K1 isolates are well-known and any such type of any such strains can be used as sources of suitable polysaccharide. Preferably the polysialic acid should have at least 2, preferably at least 5, more preferably at least 10, for instance more than 50 sialic acid units.

According to the invention, there is also provided a conjugate of a protein and the novel activated polysaccharide. The novel compound comprises a protein with at least one cysteine unit and, linked through a thioether bond to the side chain of a cysteine unit, a polysialic acid through a moiety joined at one or both terminal unit of the polysialic acid.

Where the polysialic acid derivative was a N-maleimido group the moiety will include a N-succinimidyl group, with the thioester linked at the α -carbon atom. Preferably the moiety also comprises a secondary amine, a hydrazone or an amide bond.

There is also provided in the invention a new process in which a polysialic acid is reacted with a heterobifunctional reagent having a first functional group for reaction with sulfhydryl groups and a second functional group different to the first group whereby the said second functional group
5 reacts with a terminal unit of the polysialic acid to form a covalent bond therewith and form a capable of reaction with a sulfhydryl group functional polysialic acid.

In one embodiment the second functional group is a nucleophilic group, preferably hydrazine. This is of particular utility where the polysialic
10 acid comprises an aldehyde group in the terminal unit whose carbonyl group is attacked by the nucleophilic group.

In another embodiment of the process the second functional group is electrophilic such as an N-alkoxyl carbonyl-imide such as N-hydroxysuccinimide ester or sulphosuccinimide ester, or carbodiimide. The
15 terminal group in such cases is preferably nucleophilic such as amine.

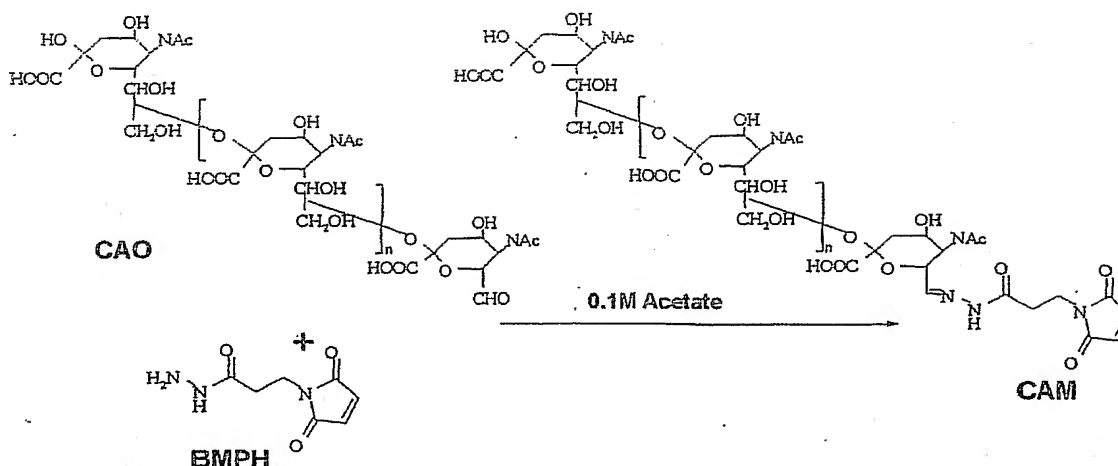
In the process it is preferred that the reagent comprises a bifunctional organic group linking the first and second functional groups. Preferably the bifunctional organic group is selected from a C₂₋₁₈-alkanediyl group, an
arylene group, an oligo peptide and an oligo(alkoxy)alkyl group.

20 Examples of suitable reagents are given above.

Most usefully the process involves a subsequent step in which the functional polysialic acid is reacted with a polypeptide or a protein having at least one free and unprotected Cys unit whereby the functional group forms a thioether linkage with the thiol group of a Cys unit to form a polysialylated
25 polypeptide or protein. The process is particularly suitable where the protein contains a single Cys unit, whereby site-controlled derivatisation is achieved.

The invention is illustrated in the accompanying examples.

30

Example 1**1.1 Synthesis**

Three separate preparations were carried out as follows:

Colominic acid aldehyde (CAO) produced according to WO-A-9222331 (100 mg, 4.4×10^{-6} mol) was dissolved into 500 μ l 0.1 M sodium acetate, to this 5 molar equivalents of *N*-[β -Maleimidopropionic acid] hydrazide (6.5 mg, 2.2×10^{-5} mol) was added. This mixture was then vortex mixed and wrapped in foil and allowed to incubate at 37°C for 2h on a rotary mixer. The polymer was then precipitated by the addition of 2 volumes (1.0 ml) of ethanol. The precipitate was collected by centrifugation (13,000 rpm 2 min) in a bench top microcentrifuge. The supernatant was discarded and the pellet dissolved in 500 μ ml 0.1 M acetate. This process was repeated a further 2 times and the final pellet dissolved in deionised water and freeze dried overnight.

1.2 Assay for Maleimide Content

In this assay cysteine is reacted with the maleimide on the polymer preventing further reaction with Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid)) which contains a disulphide which forms an intense yellow colour when it is substituted for a thiol not adjacent to an aromatic ring. Thus maleimide content can be calculated by measuring the inhibition of reaction between cysteine and Ellman's reagent assay.

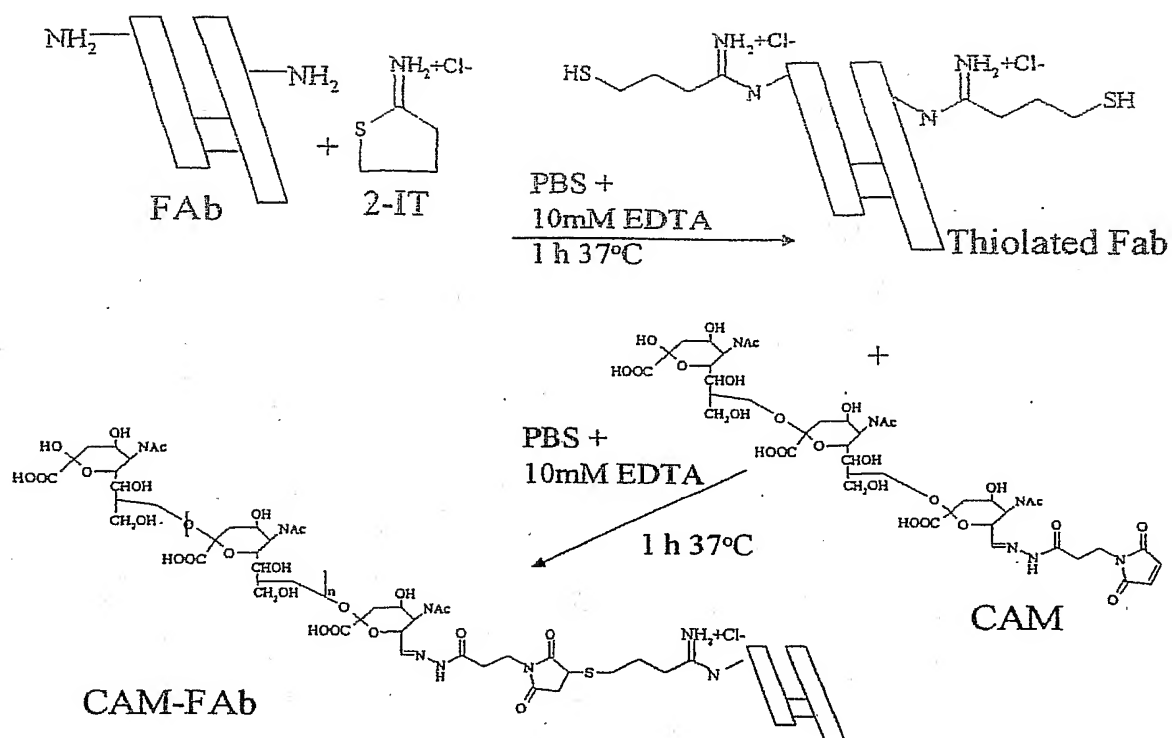
First an initial stock of cysteine at 12×10^{-3} M (0.145 mg/ml) was prepared in PBS. In a clean microtitre plate 100 μ l volume doubling dilutions from 12×10^{-3} M to 0.375×10^{-3} M were made from row B to row H. In row A 100 μ l PBS was used as a zero standard. Samples of CA and CA-maleimide (CAM) were prepared at 5 or 10 mg/ml and 100 μ l of each sample added to duplicate columns of the cysteine dilutions. In one set 100 μ l PBS without any CA was added as a control. The plate was covered and allowed to incubate at 37°C for 1 h. After this time 20 μ l of Ellman's reagent (4 mg/ml) was added to each well and the plate incubated in the dark at room temperature for 15 min. Absorbance was measured in wells at 405 nm. Standard curves were then plotted for the samples and the amount of maleimide present calculated from inhibition of the signal.

1.4 Thiolation of FAb and conjugation to CAM

In the first step a thiol group is introduced into a model protein by thiolation of an amine of lysine.

Ovine FAb (anti Desipramine /Norityrptaline, 4mg, 7.2×10^{-8} mol) was dissolved in 0.25 ml PBS + 10 mM EDTA to this was added 0.498 mg 2-iminothiolane (2-IT or Traut's reagent 50 mol equiv 3.6×10^{-6} mol) in 0.25 ml of the same buffer. The tube was wrapped in foil and left to incubate stirring end over end for 1 h at 37°C.

11



Thiolated Fab was purified from free 2-IT (Traut's reagent) by gel filtration (PD-10) and 0.5 ml fractions assayed for presence of protein (BCA assay) or thiol (Ellman's assay). The first eluting peak containing both was pooled and protein and thiols quantified.

5 1.5 Conjugation of Fab-thiol to CAM

To thiolated Fab (3.6 mg, 6.6×10^{-8} mol) in 2 ml PBS/EDTA 22.5 mg CAM was added (9×10^{-7} mol, 15 molar equiv). The tube was sealed and allowed to incubate at 37°C for 1 h whilst gently mixing. The resulting conjugate was then purified according to accepted protocols to remove free CAM. Both CA and protein content were assayed on the conjugate to calculate conjugation ratio.

Control reactions were carried out with CA as a negative control.

Several batches of CAM-Fab were prepared with various degrees of thiolation but maintaining the 15:1 ratio of CAM: Fab. Results are shown in table 1 below:

Table 1

Thiol per FAb	Conjugate ratio (CA: Fab)
1	0.53:1
2	0.9:1
5 (triplicate reaction)	1.925:1 +/- 0.19 : 1 Fab
10	3.51:1

Figure 1 shows an SDS-PAGE gel of triplicate samples and relevant controls

1.6 Conclusion

The results show that in all control wells (samples of thiolated FAb) the migration of the sample is similar to that for fresh FAb (below that of the 50 kDa marker) indicating no cross linking of FAb molecules during the process of conjugation. In the replicate lanes there is considerable band broadening with maximum intensity between the 98 and 250 kDa markers which typically indicates an increase in mass which is indicative of a polysialylated product.

1.7 Conjugation of CAM to beta galactosidase

To *E.coli* β -galactosidase (β -gal: 5.0 mg, 4.3×10^{-8} mol) in 1ml PBS 15 mg CAM was added (6.59×10^{-7} mol, 15 molar equiv). The tube was sealed wrapped in foil and allowed to incubate at room temperature for 1 h whilst gently mixing. The resulting conjugate was analysed by SDS page and then purified according to accepted protocols to remove free CAM. Samples were assayed for polymer and protein content as outlined elsewhere.

Control reactions were carried out with CA as a negative control.

1.8 Assay for Enzyme Activity

Standards from 60 μ g/ml to 3.75 μ g/ml of fresh β -galactosidase were prepared in PBS. Sample of CAM- β -gal were diluted to 60 μ g/ml in the same buffer. Enzyme activity of the conjugates was measured as follows:

In a microtitre plate, to 100 μ l of sample or standard was added 100 μ l of All-in-One β -gal substrate (Pierce). The plate was incubated at 37°C for 30 min and absorbance read at 405nm. A calibration curve was prepared from

the standards and the activity of the samples calculated from the equation for the linear regression of the curve.

1.9 Results

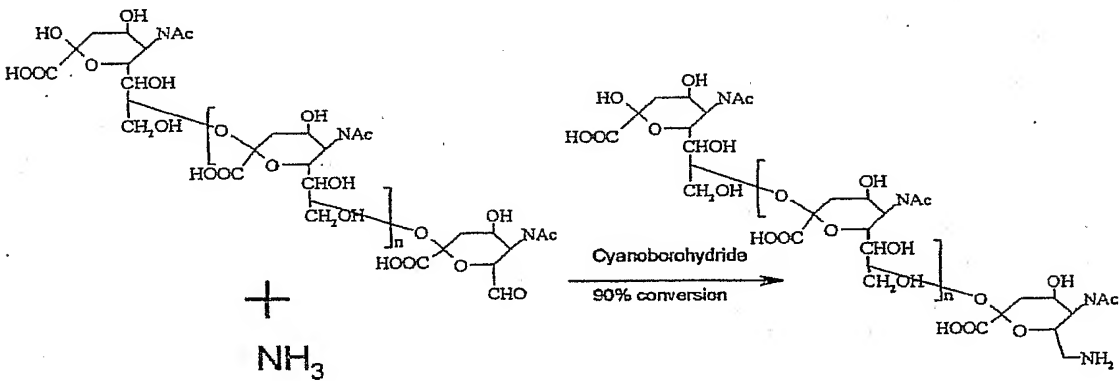
From the protein and polymer assays the conjugation ratio was determined to be 1.23 CAM:1 β -Gal. There was also a corresponding increase in apparent molecular mass from the SDS page of the samples (Fig 2). Enzyme activity in the purified sample was calculated to be 100.4 % compared to the free enzyme.

Example 2 .

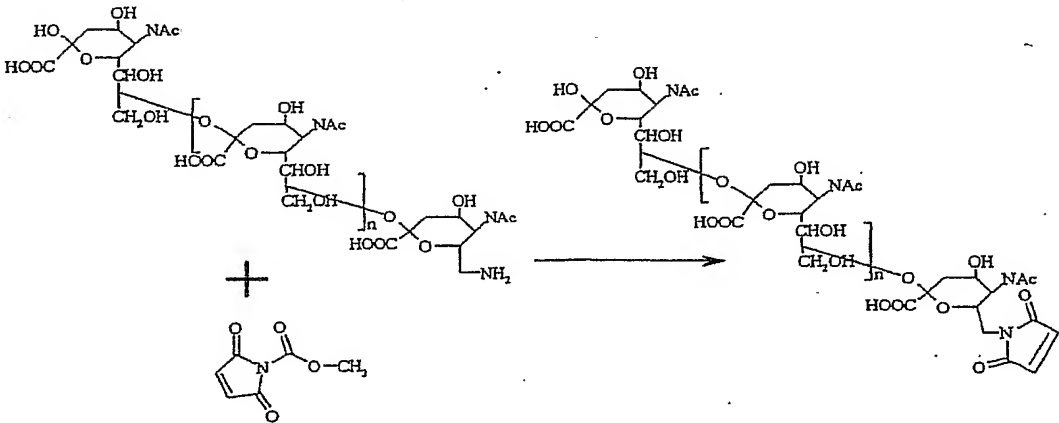
10

Synthesis Route 2

Step 1 amination of CA-aldehyde (CHO)



Step 2 introduction of maleimide ring



Synthesis

2.1. Step 1 amination of oxidised CA

Oxidised colominic acid (CAO) 10-100 mg/ml was dissolved in 2 ml of deionised water with a 300-fold molar excess of NH_4Cl , in a 50 ml tube and then NaCNBH_3 (5 M stock in 1 N NaOH(aq)) was added at a final concentration of 5 mg/ml. The mixture was incubated at room temperature for 5 days. A control reaction was also set up with colominic acid (A) instead of CAO. Product colominic acid amine derivative was precipitated by the addition of 5 ml ice-cold ethanol. The precipitate was recovered by centrifugation at 4000 rpm, 30 minutes, room temperature in a benchtop centrifuge. The pellet was retained and resuspended in 2 ml of deionised water, then precipitated again with 5 ml of ice-cold ethanol in a 10 ml ultracentrifuge tube. The precipitate was collected by centrifugation at 30000 rpm for 30 minutes at room temperature. The pellet was again resuspended in 2 ml of deionised water and freeze-dried.

2.2. Assay for amine content

The TNBS (picrylsulphonic acid or 2, 4, 6-tri-nitro-benzene sulphonic Acid) assay was used to determine the amount of amino groups present in the product.

In the well of a microtitre plate TNBS (0.5 μl of 15 mM TNBS) was added to 90 μl of 0.1 M borate buffer pH 9.5. To this was added 10 μl of a 50 mg/ml solution of CA-amide the plate was allowed to stand for 20 minutes at room temperature, before reading the absorbance at 405nm. Glycine was used as a standard, at a concentration range of 0.1 to 1mM. TNBS trinitrophenylates primary amine groups. The TNP adduct of the amine is detected.

Testing the product purified with a double cold-ethanol precipitation using the TNBS assay showed close to 90 % conversion.

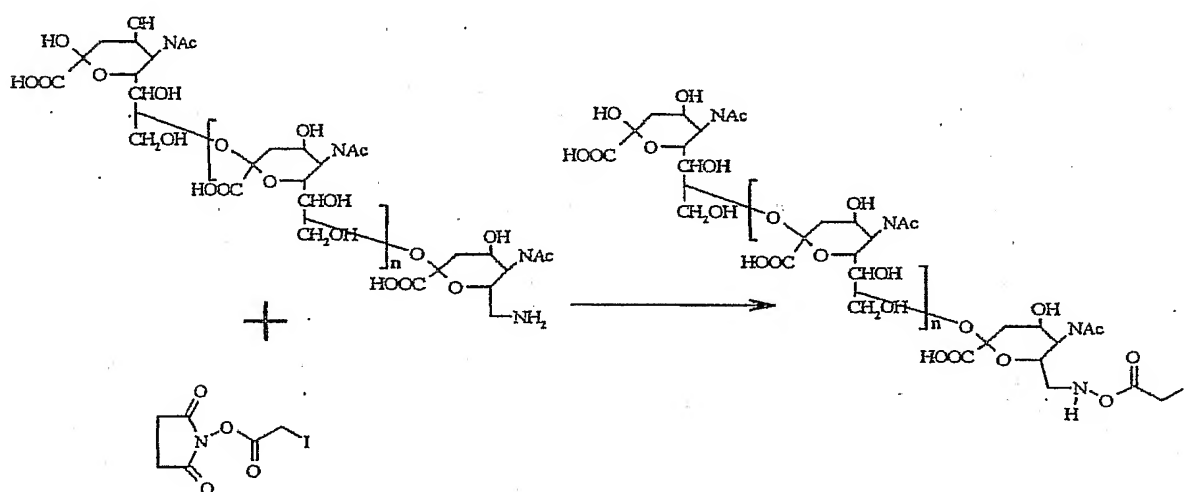
2.3. Maleimidation of CA-amine

CA-Amine (17 mg) was dissolved in 1ml deionised water, to this was added 6 mg methoxy-carbonyl-maleimide (MCM). The mixture was left to

15.

react at room temperature for 30 min. To the sample 100 μ l water and 200 μ l acetonitrile was added and then incubated at room temperature for 4 h, after which 300 μ l CHCl_3 was added, the tube shaken and the aqueous fraction collected. Then the fraction was purified over a PD-10 column to remove small molecules. The eluate was freeze dried and assayed for maleimide content. The molar concentration of maleimido was 44 mol %.

Example 3: Preparation of Iodoacetate derivative of colominic acid (CAI).



3.1 Synthesis

To 40 mg colominic acid amine (85 mol % amine) as (described in Example 2.1) dissolved in 1ml of PBS pH 7.4 was added 5 mg of N-succinimidyl iodoacetate (SIA). The mixture was left to react for 1h at 37°C, after which excess SIA was removed by gel filtration over a 5ml Hightrap™ Desalting column (AP Bioscience) eluted with PBS. 0.5 ml fractions were collected from the column and samples from each fraction tested for colominic acid content (resorcinol assay) and reactivity with cysteine indicating iodide (Ellman's Assay). Fractions positive for both iodide and CA were pooled.

3.2 Conjugation of CAI to β -galactosidase

To *E.coli* β -galactosidase (5.0 mg, 4.3×10^{-8} mol) in 1ml PBS 15 mg CAI was added (6.59×10^{-7} mol, 15 molar equiv). The tube was sealed

wrapped in foil and allowed to incubate at room temperature for 1 h whilst gently mixing. The resulting conjugate was analysed by SDS page and then purified according to accepted protocols to remove free CAI. Samples were assayed for polymer and protein content as outlined elsewhere.

- 5 Control reactions were carried out with CA as a negative control. All samples were analysed for β -gal activity as example 1.8 above.

3.3 Conclusions

Fractions 3-6 were positive for both polymer and iodoacetate and were pooled. The SDS page (4-12 % Bis/Tris gel; Figure 2) showed an
10 increase in apparent molecular mass for samples incubated with the iodoacetamide derivative but not with control polymer. From the protein and polymer assays the conjugation ratio was determined to be 1.63 CAI:1 β -gal. β -gal activity was calculated to be 100. 9 % for the conjugated sample, compared to the free enzyme.

15 Example 4

4.1 Synthesis: Non-reducing end activation with 4(4-N-maleimidophenyl) butyric acid hydrazide (CA-MBPH) and 3-(2-pyridyldithio) propionyl hydrazide (CA-PDPH)

The preparations were made as follows:

- 20 Colominic acid aldehyde (CAO; 22.7kDa, Camida, Ireland) produced according to WO-A-9222331 (73 mg for MBPH, tube A; 99.3 mg for PDPH; tube B) was dissolved individually into 800 μ l 0.1 M sodium acetate (pH 5.5). To tube A 15 mg of MBPH (15: 1 linker : CA ratio) dissolved in 200 μ l of DMSO was added. To tube B 15 mg (15: 1 linker : CA ratio) of PDPH
25 dissolved in 200 μ l of DMSO was added. The pH was adjusted to 5.5 in each case for each reaction vessel. These mixtures were then vortex mixed and wrapped in foil and allowed to incubate at 37°C for 2h on a orbital mixer. Each polymer solution was purified by gel filtration (PD 10 column) eluting with PBS pH 7.4 and the 1ml fraction containing CA (by resorcinol assay)
30 collected. These sample were freeze dried overnight and assayed for maleimide content as described in example 1.2.

4.2 Synthesis: Reducing end activation with 4(4-N-maleimidophenyl)butyric acid hydrazide (MBPH-CA), (2-pyridyldithio)propionyl hydrazide (PDPH-CA) and N- β -Maleimidopropionic acid hydrazide (BMPH-CA)

5 The preparations were carried out as follows all at molar ratios of 25:1 linker to CA:

Colominic acid (CA; 22.7kDa; 73 mg for MBPH, tube A; 99.3 mg for PDPH; tube B and 76.6 mg for BMPH; tube C) was dissolved individually into 800 μ l 0.1 M sodium acetate (pH 5.5) separately. To tube A, 25 mg of MBPH (dissolved in 200 μ l of DMSO), to tube B 25 mg of PDPH (dissolved in 200 μ l of DMSO) and to vial C 25 mg of BMPH (dissolved in 200 μ l of sodium acetate buffer) were added. The pH of the reaction mixture was adjusted to 5.5. Each mixture was then vortex mixed, wrapped in foil and allowed to incubate at 37°C for 72h on a orbital mixer. Each polymer solution was purified by gel filtration (PD 10 column) eluting with PBS pH 7.4 and the 1ml fraction containing CA (by resorcinol assay) collected. These sample were freeze dried overnight and assayed for maleimide content as described in example 1.2.

4.3 Results

20 The molar concentration of maleimido was 49.0 and 35.0 mol % for MBPH and PDPH respectively on the non-reducing (highly reactive) end. The molar concentration of maleimido was 41.5, 32.5 and 48.3 mol % for MBPH, PDPH and BMPH respectively on the reducing end (weakly reactive). The values on the reducing end are average of two values in each case.

25 **4.4 Conjugation of β -galactosidase (β -gal) to Maleimide activated colominic acids (reducing end and non-reducing end)** To β -gal (1.0 mg; 1 ml in PBS) in separate tubes, a 15 molar excess of each maleimide activated CA (MBPH, PDPH and BMPH on non-reducing or reducing end, from above examples) was added separately. Each tube was sealed and allowed to incubate at 37°C for 1 h whilst gently mixing. The resulting conjugate was then purified according to accepted protocols to remove free

30

CLAIMS

1. A compound comprising a polysaccharide having at least two sialic acid units joined to one another and having a pendant moiety linked to at least one terminal unit derived from a sialic acid unit which includes a functional group selected from N-maleimide groups, vinylsulphone groups, N-iodoacetamide groups and orthopyridyl disulphide groups.

2. A compound according to claim 1 in which the pendant moiety is linked at the reducing terminal unit of the polysaccharide.

3. A compound according to claim 1 or claim 2 in which the moiety is linked at the non-reducing terminal unit of the polysaccharide.

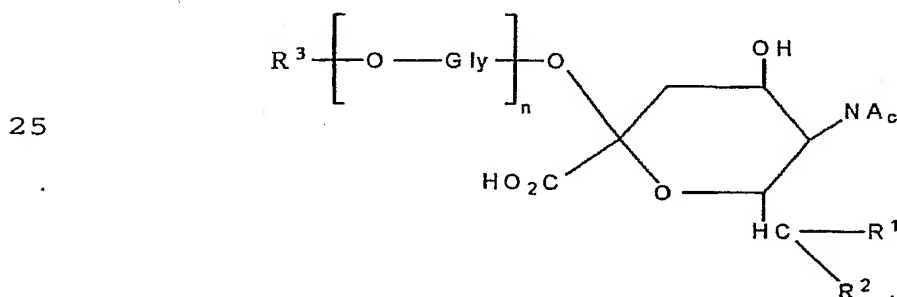
4. A compound according to any preceding claim in which the moiety comprises an alkanediyl group and/or an arylene group and a linkage optionally in combination with a oxalkylene or oligooxa-alkylene group which is a secondary amine linkage, a hydrazone, an alkyl hydrazide linkage or a peptide linkage.

5. A compound according to any preceding claim in which the functional group is N-maleimido.

6. A compound according to any preceding claim in which the polysaccharide is a polysialic acid.

7. A compound according to claim 6 in which the polysaccharide consists substantially only of sialic acid units.

8. A compound according to claim 1 which has the formula



in which one of the following groups of definitions apply:

30 i) R^1 is H or $-\text{CHOHCH}_2\text{OH}$, R^2 is OH and R^3 is either

-CH₂CHR⁴R⁵ or -CH(CH₂OH)CHR⁴R⁵ in which R⁴ and R⁵ together represent =N-NR⁶ or R⁴ is H and R⁵ is -NR⁶R⁷ in which R⁶ is an organic group comprising the said functional group or is H, and R⁷ is H or R⁶ and R⁷ together are a 1,3-but-2-enedioyl group;

- 5 ii) R¹ and R² together represent =N-NR⁶ or R¹ is H and R² is -NR⁶R⁷ in which R⁶ is an organic group comprising the said functional group or is H, and R⁷ is H or R⁶ and R⁷ together are a 1,3-but-2-enedioyl group;

R³ is H;

Gly-O is a glycosyl (saccharide) group;

10 n is 0 or more; and

Ac is acetyl.

9. A compound according to claim 8 in which each Gly is a sialic acid unit.

15 10. A compound comprising a protein with at least one free cysteine unit and, linked through a thioester bond to the side chain of the cysteine unit, with a polysialic acid, through a moiety joined at one or each terminal units of the polysialic acid.

11. A compound according to any preceding claim in which the polysaccharide has at least 10 saccharide units.

20 12. A compound according to claim 11 which has at least 50 saccharide units.

13. A compound according to claim 11 or claim 12 in which the saccharide units are sialic acid units 2, 8 and/or 2, 9 linked to one another.

25 14. A process in which a polysaccharide having at least two sialic acid units joined to one another and comprising at least one terminal unit which is derived from a sialic acid unit is reacted with a heterobifunctional reagent having a first functional group selected from N-maleimido groups, vinylsulphone groups, N-iodoacetamide groups and orthopyridyl disulphide groups and a second functional group different from the first group whereby
30 the said second functional group reacts with a terminal sialic acid derivative

unit to form a covalent bond therewith and form a functional polysaccharide suitable for selective conjugation to a thiol group.

15. A process according to claim 14 in which the said second functional group is a nucleophilic group.

5 16. A process according to claim 15 in which the nucleophilic group is hydrazine.

17. A process according to claim 14 in which the terminal unit of the polysaccharide has a carbonyl group which reacts with said nucleophilic group.

10 18. A process according to claim 14 in which the said second functional group is an electrophilic group.

19. A process according to claim 18 in which the electrophilic group is an N-alkoxycarbonyl-imide or carbodiimide moiety.

15 20. A process according to claim 18 or 19 in which the terminal unit of the polysaccharide has an amine group which reacts with said electrophilic group.

21. A process according to claim 20 in which a peptide or urethane linkage is formed.

20 22. A process according to any of claims 14 to 21 in which the reagent comprises a bifunctional organic group linking the first and second functional groups.

23. A process according to claim 22 in which the bifunctional organic group is selected from a C₂₋₁₈-alkanediyl group, an arylene group, an oligo peptide and an oligo(alkoxy)alkyl group.

25 24. A process according to any of claims 14 to 23 in which the first functional group is a N-maleimide group.

25. A process according to claim 14 in which the reagent has the general formula



30 in which:

X is a N-maleimido, N-iodoacetamido, S-vinylsulphonyl or S-orthopyridyldisulphide group,

R is alkane-diyl, arylene or aralkylene alkarylene, alkylene-oxaalkylene, or alkylene-oligooxa-alkylene or alkyl-oligopeptidyl-alkyl group;

5 and

Y is a hydrazide, amine or N-hydroxysuccinimide group.

26. A process according to any of claims 14 to 25 in which the polysaccharide has at least 10 sialic acid units.

27. A process according to claim 26 in which the polysaccharide
10 has at least 50 sialic acid units.

28. A process according to claim 26 or 27 in which the sialic acid units are 2-8 and/or 2-9 linked to one another.

29. A process according to any one of claims 14 to 28 in which the maleimido-functional polysialic acid is reacted with a polypeptide or a protein
15 having at least one free unprotected Cys unit whereby the maleimide group forms a thioether linkage with the thiol group of a Cys unit to form a polysialylated polypeptide or protein.

30. A process in which a compound according to any of claims 1 to 6 is reacted with a polypeptide or a protein having at least one free and
20 unprotected Cys unit whereby the said functional group forms a thioether linkage with the thiol group of a Cys unit to form a conjugate of the polysaccharide with the polypeptide or protein.